Molecular Cloning of a Novel Hyaluronan Receptor That Mediates Tumor Cell Motility

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Abstract. A cDNA encoding a unique hyaluronan receptor has been molecularly cloned from a λGT11 3T3 cDNA expression library. Immunoblot analyses of cell lysates, using antibodies to peptides encoded in the cDNA, specifically react with a 58-kD protein. This protein is regulated by the mutant H-*ras* gene in cells containing a metallothionein promoter H-*ras* hybrid gene. Further, antibodies to peptide sequences encoded in the cDNA block the increase in locomotion resulting from induction of the mutant H-*ras* gene in this cell line. In a transblot assay, the bacterially expressed protein binds to biotinylated hyaluronan. Antibodies to peptides encoded in the cDNA react in immunoblot assays with the 58- and 52-kD proteins of a novel hyaluronan receptor complex previously implicated in cell locomotion. Furthermore, antibodies specific to the 58- and 52-kD proteins, which block *ras*-induced locomotion, also cross-react with the expressed, encoded protein. The gene product described here appears to be a new type of hyaluronan receptor that is involved in cell locomotion. It is named RHAMM, an acronym for receptor for hyaluronan-mediated motility.

THE transforming oncogene H-*ras* has been reported to promote cell locomotion (17), although the regulatory mechanisms remain unknown. Several observations suggest that when this oncogene promotes locomotion, the mechanisms are complex and involve, at least, the release of autocrine motility factor(s) (14, 20), growth factors (14), and the glycosaminoglycan hyaluronan (HA)[1] (20, 34). In particular, HA appears to function as an autocrine mechanism for stimulating maximal locomotion in *ras*-transformed cells (34). Further, it is also required for the ability of an autocrine motility-stimulating factor to promote breast carcinoma cell locomotion (20). We have shown that HA-promoted, *ras*-transformed cell locomotion requires the presence of a novel hyaluronan receptor complex termed HARC (34). This complex of proteins occurs at the cell surface or is released as soluble proteins of 72, 68, 58, and 52 kD (32). The complex is tightly regulated in vitro (30) and expressed on the leading lamellae and perinuclear region only on rapidly locomoting cells (29, 31). Both polyclonal and monoclonal antibodies (pAbs and mAbs, respectively) prepared against this complex block cell locomotion regulated by mutant *ras* (34). In a recent study, we have shown that these blocking mAbs are specific to the 58- and 52-kD proteins, that these proteins are isoforms of each other, and, further, that these proteins are the HA-binding component of HARC (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication).

We have used the blocking antibodies specific to the 58- and 52-kD HARC proteins to screen a λGT11 3T3 cDNA expression library. We describe here the molecular cloning of a cDNA encoding a unique protein that is regulated by mutant H-*ras*, that is essential for the locomotion of these *ras*-transformed cells, and that binds to HA. Further, antibodies to peptides encoded in the cDNA cross-react with the 58- and 52-kD proteins of HARC, while conversely blocking mAbs to the 58-kD HARC protein cross-react with the expressed, encoded protein. This is the first molecularly characterized protein identified as a requirement for the locomotion of *ras*-transformed cells. Further, our data suggest that this novel protein encodes a new HA receptor. It is therefore referred to by the acronym RHAMM for receptor for HA-mediated motility.

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1. *Abbreviations used in this paper*: HA, hyaluronan; HARC, hyaluronan receptor complex; pAb, polyclonal antibody; RHAMM, receptor for HA-mediated motility.

Materials and Methods

Antibodies

pAbs and mAbs to HARC were prepared as described previously (31, 32, 34). All antibodies were purified by affinity chromatography on HARC-

Sepharose and/or protein A-Sepharose. mAb 3T3-3, which blo cks fibroblast locomotion, was originally used to screen the cDNA library. mAb 3T3-5 is shown here to recognize proteins which occur in two isoforms (58 and 52 kD) in SDS immunoblots of lysates of *ras*-transformed fibroblasts and has previously been shown to block HA-promoted locomotion of these fibroblasts (34). Mab 3T3-7, which was used for immunofluorescence, reacts specifically with the 52–58-kD protein of HARC, as shown here by SDS immunoblot analyses, but does not block HA-promoted locomotion (34). Synthetic peptides mimicking two amino acid sequences in the deduced sequence (peptide I, nucleotide sequence 372–435 [corresponding to a portion of the repeated sequence] and peptide II, nucleotide sequence 804–864 [Fig. 1]) were produced by the Peptide Analysis Core of the University of Alabama Cancer Center, Birmingham, AL. The peptides were purified by HPLC and characterized by amino acid composition analyses. Antibodies to the peptides were raised in rabbits as described (31, 32). These antibodies were purified by affinity chromatography on columns of the appropriate peptides coupled to CNBr-activated Sepharose and the specificity of purified antipeptide antibodies to the respective peptide was confirmed by dot blot assays. Preimmune antibodies were obtained from each rabbit or mouse before immunization, purified as described above and used as controls.

Cloning and DNA Sequencing

A 3T3-cDNA library in λgt11 was obtained from Clonetech (Palo Alto, CA) and initially screened with both pAb and mAb (3T3-3) to HARC. One clone, which gave a positive signal upon repeated screening with the antibodies, had a 1.9-kb insert that was subcloned into pUC18 and M13. A restriction map was constructed using the enzymes (Un. States Biochem. Corp., Cleveland, OH) indicated in Fig. 1. Nucleotide sequences were determined by the dideoxy chain termination method using Sequenase I and Sequenase II kits (Un. States Biochem. Corp.). Digestion of the insert with AccI yielded a 0.7 kb fragment which was radiolabeled with a Random priming kit (Bethesda Research Laboratories, Bethesda, MD) and was used to rescreen the cDNA library at high stringency, resulting in the isolation of a 2.9-kb insert. The insert was isolated after digestion of the λphage with EcoR1 and ligated into pUC18 and M13 for sequencing. Sequence analysis and comparisons were conducted using IBI/Puskell sequence analysis software and NBRF database (version 18; reference 4). Additional homology searches were conducted with NBRF (6/90) and EMBL (5/90) using FASTA and word search programs in GCG software.

Locomotion Assays

A 10T1/2 cell line containing an inducible EJ-*ras* metallothionein-neomycin hybrid gene (designated 212; reference 26) was used for quantitating the effect of both pAb to synthetic peptide and mAb to HARC on cell locomotion in response to 0.001 μg/ml of HA as previously described (34). Antibodies (mAb 3T3.5, 1 μg/ml, or pAb peptide II, 1 μg/ml) were added to zinc-induced (24 h) cells for 0.5 h, and then exposed to 0.001 μg/ml of HA and filmed for a total of 2 h in the presence of both HA and either of the above antibodies. To assess specific effects of the antibodies, they were preincubated for 1 h at 4°C with 50 μg of HARC proteins before their addition to locomotion assays as above. Addition of these proteins by themselves had no effect on cell locomotion (results not shown). Data were analyzed with a Dynacell program (Carl Zeiss, Inc., Thornwood, NY) that used Fourier analysis of cell locomotion to derive a cell motility index (17).

Immunofluorescence of Live Cells

Cells were grown on glass coverslips for 24 h after their subculture so that monolayers were subconfluent. Cells were then incubated with either Ca^{++}, Mg^{++}-free Hanks' solution or 0.25% trypsin in Ca^{++}, Mg^{++}-free Hanks' solution for 3 min. Primary antibody (pAb II, nucleotide sequence 804–864, or rabbit IgG) was then added at 1 μg/ml in defined DMEM to cultures and incubated for 2–3 h at 37°C. Media was gently aspirated from cultures which were then washed and incubated with rhodamine-labeled goat anti-rabbit IgG (Sigma Chem. Co., St. Louis, MO; 1:100 dilution) for 1 h at room temperature. Cells were washed again, and then lightly fixed in freshly made 3% paraformaldehyde. Fixed monolayers were viewed on both an IM35 microscope (Carl Zeiss, Inc.) equipped with epifluorescence and also on a Nikon confocal laser scanning microscope. For confocal microscopy, only the first 3 μm of the culture media-facing surface of cells was photographed.

Immunofluorescence of Fixed Cells

Double immunofluorescence studies of zinc-induced 212 cells were performed as described (30, 31) using mAb 3T3-7 to HARC and pAb to peptide II (nucleotide sequence 804–864). Monolayers were fixed in freshly prepared 3% paraformaldehyde for 10 min, and then incubated with 0.1 M glycine to quench autofluorescence. Fixed monolayers were washed, and then incubated with 1 μg/ml of the above antibodies overnight at 4°C. The monolayers were again washed, and then incubated with fluorescein-labeled goat anti-mouse IgG (to detect mAb 3T3-7) and rhodamine-labeled goat anti-rabbit IgG (to detect pAb to peptide I). These were purchased from Sigma Chem. Co. and used at 1:1,000-fold dilution. Processed monolayers were examined with an IM35 microscope (Carl Zeiss, Inc.) equipped with epifluorescence utilizing nonoverlapping filters of 510–560 nm (for rhodamine) and 450–490 nm (for fluorescein). Lack of bleedthrough was confirmed by examination of single immunofluorescence samples with both filters (data not shown).

SDS Immunoblots

Immunoblot assays were conducted on isolated soluble HARC proteins (32) or cell lysates prepared from H-*ras*-transfected fibroblasts exposed to either zinc sulfate or buffer alone for 24 h. The cells were treated with lysis buffer containing 25 mM Tris, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, and protease inhibitors (32). Proteins were fractionated by SDS-PAGE on 12.5% polyacrylamide gels (32) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Rockville Center, NY). Additional protein binding sites were blocked with 0.5% defatted milk in TBS (34) and the membrane was incubated with primary antibodies (1 μg IgG/ml of TBS containing 0.5% defatted milk) for 12 h at 4°C on a gyratory shaker (Stovall, Greensboro, NC). The mAb to p21 *ras* (pan. *ras*-10) was purchased from Du Pont Co., Wilmington, DE. The washed membranes were incubated with HRP-labeled goat IgG as a secondary antibody for 20 to 60 min at room temperature. After washing, the membranes were developed with peracid followed by luminol and an enhancer following the instructions of the manufacturer (ECL; Amersham Corp., Arlington Heights, IL). The oxiacid product was detected by immediately exposing with Kodak X-Omat Xac-5 film.

Isolation of RNA and Northern Assays

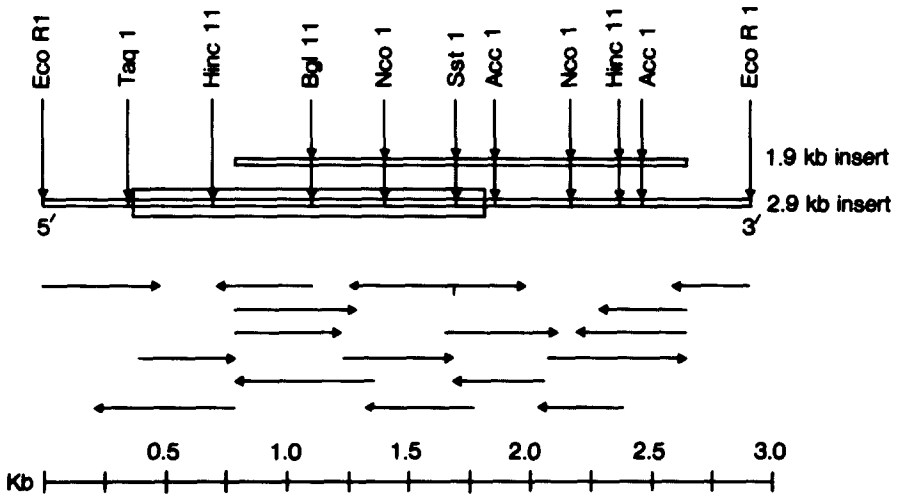
Confluent cultures of *ras*-transfected cells were exposed to zinc sulfate or to buffer alone (34) for 24 h. Total RNA was extracted according to Choy et al. (6). 60 μg of RNA was electrophoresed on 1% agarose gels and transblotted onto Nytran Nylon membranes. The mRNA transcript for the encoded protein was detected using ^{32}P-labeled 1.7-kb fragment of the cDNA that contained the open reading frame. Blots were reprobed with ^{32}P-glyceraldehyde-3-phosphate dehydrogenase cDNA to control for RNA loading.

Expression of RHAMM in Bacteria

Oligonucleotide primers corresponding to the second initiation codon and the stop codon were prepared and used to amplify the complete open reading frame by polymerase chain reaction. The second initiation codon was chosen since the size of the protein generated by *N*-glycanase digestion of the 58-kD protein closely matched this (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication). Polymerase chain reaction generated a 1.3-kb DNA fragment which was cloned into the PGEX-2T expression vector (22) and transformed into *Escherichia coli* (JM101). Induction of protein expression in cultures of transformed *E. coli* with 0.2 mM isopropyl-β-D-thiogalactopyranoside resulted in the production of an insoluble recombinant glutathione-S-transferase fusion protein. Insoluble fusion protein was solubilized by the addition of 4 M urea, 0.05 M Tris, 1 mM EDTA, 1% Triton X-100, pH 8.0, followed by gradual removal of the urea by dialysis into 2 M urea, 0.05 M Tris, 1 mM EDTA, 1% Triton X-100, pH 8.0, and then PBS, 1% Triton X-100, pH 7.4. Solubilized fusion protein was subsequently purified by affinity chromatography on glutathione-agarose (22).

HA Binding Assays

Lysates (25 μg) from induced bacteria containing either the parental PGEX-2T vector or the PGEX-2T vector with RHAMM DNA were electrophoresed on 10% SDS-PAGE and transblotted onto nitrocellulose membranes.

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MET Gln Ile Leu Thr Glu Arg Leu Ala Leu Glu Arg Gln Glu Tyr Glu Lys Leu Gln
ATG CAG ATC CTG ACA GAG AGG CTG GCT CTG GAA AGG CAA GAA TAT GAA AAG CTG CAA      57

Gln Lys Glu Leu Gln Ser Gln Ser Leu Leu Gln Gln Glu Lys Glu Leu Ser Ala Arg
CAA AAA GAA TTG CAA AGC CAG TCA CTT CTG CAG CAA GAG AAG GAA CTG TCT GCT CGT  114

Leu Gln Gln Gln Leu Cys Ser Phe Gln Glu Glu MET Thr Ser Glu Lys Asn Val Phe
CTG CAG CAG CAG CTC TGC TCT TTC CAA GAG GAA ATG ACT TCT GAG AAG AAC GTC TTT  171

Lys Glu Glu Leu Lys Leu Ala Leu Ala Glu Leu Asp Ala Val Gln Gln Lys Glu Glu
AAA GAA GAG CTA AAG CTC GCC CTG GCT GAG TTG GAT GCG GTC CAG CAG AAG GAG GAG  228

Gln Ser Glu Arg Leu Val Lys Gln Leu Glu Glu Glu Thr Lys Ser Thr Ala Glu Gln
CAG AGT GAA AGG CTG GTT AAA CAG CTC GAA GAG GAA ACG AAG TCA ACT GCA GAA CAA  285

Leu Thr Arg Leu Asp Asn Leu Leu Arg Glu Lys Glu Val Glu Leu Glu Lys His Ile
CTG ACG CGG CTG GAC AAC CTG CTG AGA GAG AAA GAA GTT GAA CTG GAG AAA CAT ATT  342

Ala Arg His Ala Gln Ala Ile Leu Ile Ala Gln Glu Lys Tyr Asn Asp Thr Ala Gln
GCT CGT CAA GCC CAA GCC ATC TTG ATT GCA CAA GAG AAG TAT AAT GAC ACA GCA CAG  399

Ser Leu Arg Asp Val Thr Ala Gln Leu Glu Ser Val Gln Glu Lys Tyr Asn Asp Thr
AGT CTG AGG GTC GTC ACT GCT CAG TTG GAA AGT GTG CAA GAG AAG TAC AAT GAC ACA  456

Ala Gln Ser Leu Arg Asp Val Thr Ala Gln Leu Glu Ser Glu Gln Glu Lys Tyr Asn
GCA CAG AGT CTG AGG GAC GTC ACT GCT CAG TTG GAA AGT GAG CAA GAG AAG TAC AAT  513

Asp Thr Ala Gln Ser Leu Arg Asp Val Thr Ala Gln Leu Glu Ser Glu Gln Glu Lys
GAC ACA GCA CAG AGT CTG AGG GAC GTC ACT GCT CAG TTG GAA AGT GAG CAA GAG AAG  570

Tyr Asn Asp Thr Ala Gln Ser Leu Arg Asp Val Thr Ala Gln Leu Glu Ser Val Gln
TAC AAT GAC ACA GCA CAG AGT CTG AGG GAC GTC ACT GCT CAG TTG GAA AGT GTC CAA  627

Glu Lys Tyr Asn Asp Thr Ala Gln Ser Leu Arg Asp Val Thr Ala Gln Leu Glu Ser
GAG AAG TAC AAT GAC ACA GCA CAG AGT CTG AGG GAC GTC ACT GCT CAG TTG GAA AGC  684

Tyr Lys Ser Ser Thr Leu Lys Glu Ile Glu Asp Leu Lys Leu Glu Asn Leu Thr Leu
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Thr Ala Glu Ser Thr Asn Gln Glu Tyr Ala Arg Met Val Gln Asp Leu Gln Asn Arg
ACA GCT GAG AGC ACA AAT CAA GAA TAT GCA AGG ATG GTT CAA GAT TTG CAG AAC AGA  855

Ser Thr Leu Lys Glu Glu Glu Ile Lys Glu Ile Thr Ser Ser Phe Leu Glu Lys Ile
TCA ACC TTA AAA GAA GAA GAA ATT AAA GAA ATC ACA TCT TCA TTT CTT GAG AAA ATA  912

Thr Asp Leu Lys Asn Gln Leu Arg Gln Gln Asp Glu Asp Phe Arg Lys Gln Leu Glu
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Glu Lys Gly Lys Arg Thr Ala Glu Lys Glu Asn Val Met Thr Glu Leu Thr Met Glu
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Ile Asn Lys Trp Arg Leu Leu Tyr Glu Glu Tyr Glu Lys Thr Lys Pro Phe Gln Gln
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Gln Leu Asp Ala Phe Glu Ala Glu Lys Gln Ala Leu Leu Asn Glu His Gly Ala Thr
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Leu Lys Gln Lys Ile Lys His Val Val Lys Leu Lys Asp Glu Asn Ser Gln Leu Lys
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Ser Glu Val Ser Lys Leu Arg Ser Gln Leu Val Lys Arg Lys Gln Asn Glu Leu Arg
TCG GAG GTG TCA AAA CTC CGA TCT CAG CTT GTT AAA AGG AAA CAA AAT GAG CTC AGA 1311

Leu Gln Gly Glu Leu Asp Lys Ala Leu Gly Ile Arg His Phe Asp Pro Ser Lys Ala
CTT CAG GGA GAA TTA GAT AAA GCT CTG GGC ATC AGA CAC TTT GAC CCT TCC AAG GCT 1368

Phe Cys His Ala Ser Lys Glu Asn Phe Thr Pro Leu Lys Glu Gly Asn Pro Asn Cys
TTT TGT CAT GCA TCT AAG GAG AAT TTT ACT CCA TTA AAA GAA GGC AAC CCA AAC TGC 1422

Cys
TGC TGA
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Figure 1. Restriction map and sequence of the complete 2.9-kb cDNA clone encoding the 52–58 HARC protein(s). Blocking pAbs and mAbs to HARC were prepared and used to screen a λgt11 3T3 cDNA expression library (Clonetech). A restriction map was constructed using the enzymes (Un. States Biochem. Corp.) indicated in the restriction map. The open reading frame of the clones is boxed. The sequencing strategy is shown below the cDNA clones. Both antibodies to peptides encoded in the cDNA (*underlined sequences*) and a radiolabeled ACCI fragment of the 1.9-kb insert were used to isolate the 2.9 complete cDNA. The amino acid sequences are shown above the DNA sequences. Two possible initiation codons are indicated with highlighted letters. Potential *N*-glycosylation sites are marked with asterisks and possible signal sequences are underlined with broken lines. The stop codon is indicated by highlighted letters. The sequence data are available from EMBL/GenBank/DDBJ under accession No. X-64550.

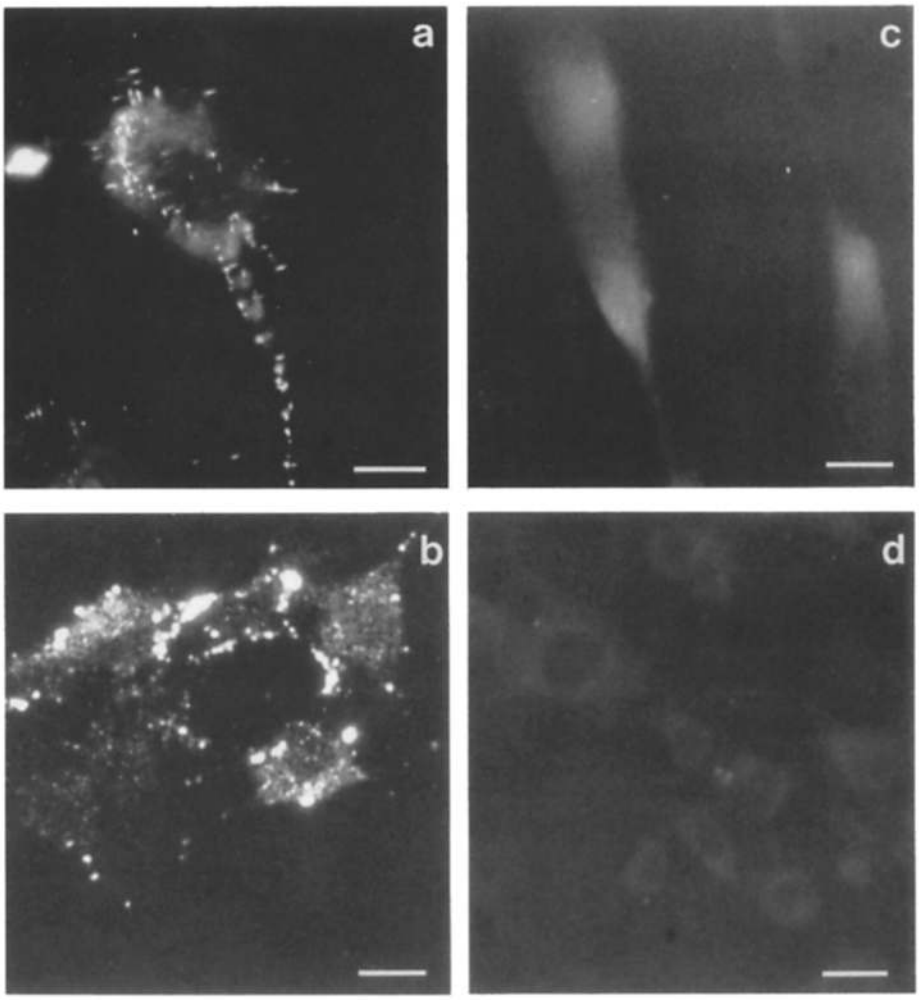

Figure 2. Immunofluorescent localization of encoded protein to the surface of live cells. To demonstrate whether or not encoded protein localized to the cell surface, H-*ras*-expressing cell monolayers were processed with (*a*) pAb to peptide II. (*b*) As in *a* but photographed with a confocal microscope. (*c*) Cells were treated lightly with trypsin before staining with primary antibody. (*d*) Cells were incubated with nonimmune rabbit IgG. Primary antibodies were visualized with RITC-conjugated goat anti-rabbit IgG and localized RHAMM to the cell surface. Trypsin treatment abolished staining and samples incubated with nonimmune sera did not exhibit staining. Bars: (*a* and *c*) 17.2 μm; (*b*) 38 μm; (*d*) 43 μm.

Nitrocellulose was blocked by incubating with 5% defatted milk in PBS for 1 h followed by incubation in the presence of biotinylated HA (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication). Nitrocellulose was washed for 1 h in TBS containing 0.05% Tween and bound HA was detected by incubation with streptavidin-HRP (1:1,000 dilution; Sigma Chem. Co.), followed by visualization with chemiluminescence (ECL; Amersham Corp.).

Results

Isolation of a cDNA Encoding RHAMM

A clone containing a 1.9-kb insert was isolated by screening a 3T3 cell cDNA expression library in λgt11 with both a mAb (designated 3T3-3) and a pAb to HARC. Sequencing of the insert revealed an open reading frame corresponding to a 340-amino acid residue of a COOH-terminal protein segment that did not include an initiation codon (Fig. 1). Additional clones coding for the same protein were isolated by rescreening the library with a 0.7-kb radiolabeled AccI restriction fragment of the 1.9-kb cDNA and with pAb to synthetic peptides mimicking segments of the deduced sequence (peptides I and II; Fig. 1). A clone containing a 2.9-kb insert was positive in both screenings and was further characterized (Fig. 1). Restriction mapping and sequencing of this insert demonstrated that it contained a complete open reading frame and the original 1.9-kb cDNA sequence in its central region (Fig. 1). The sequence was unique and did not bear significant homology to other proteins registered in NBRF or EMBL data banks or to factors known to be involved in *ras*-regulated locomotion (14). Like previously characterized proteins such as p53 (36), it contained two possible initiation codons, encoding proteins of either 52.2 or 46.7 kD, respectively (Fig. 1). The encoded protein was rich in glutamic acid, lysine, glutamine, and leucine. It had a Pi of 5.2, was hydrophillic, and most of the polypeptide was predicted to occur as an alpha helix by Chou-Fasman analyses (5). The most notable feature of the deduced sequence was a 21-amino acid stretch (which corresponded to peptide I, underlined in Fig. 1) that was repeated five times near the NH_2 terminus. The predicted protein contained eight putative *N*-glycosylation sites, five of which were concentrated within the repeated motif. The protein also contained clusters of positively charged amino acids throughout the open reading frame. It did not encode a hydrophobic sequence long enough to span the plasma membrane and possible signal sequences following either initiation codon were weak (Fig. 1).

The Encoded Protein Occurs at the Cell Surface

The encoded protein occurred at the cell surface as demonstrated by positive immunofluorescent staining for RHAMM using live cells (Fig. 2, *a*, and *b*). Further, the majority of staining occurred on cell processes and at the media surface of cells as demonstrated by optically "sectioning" cells using a confocal microscope. Staining using pAb to peptide II en-

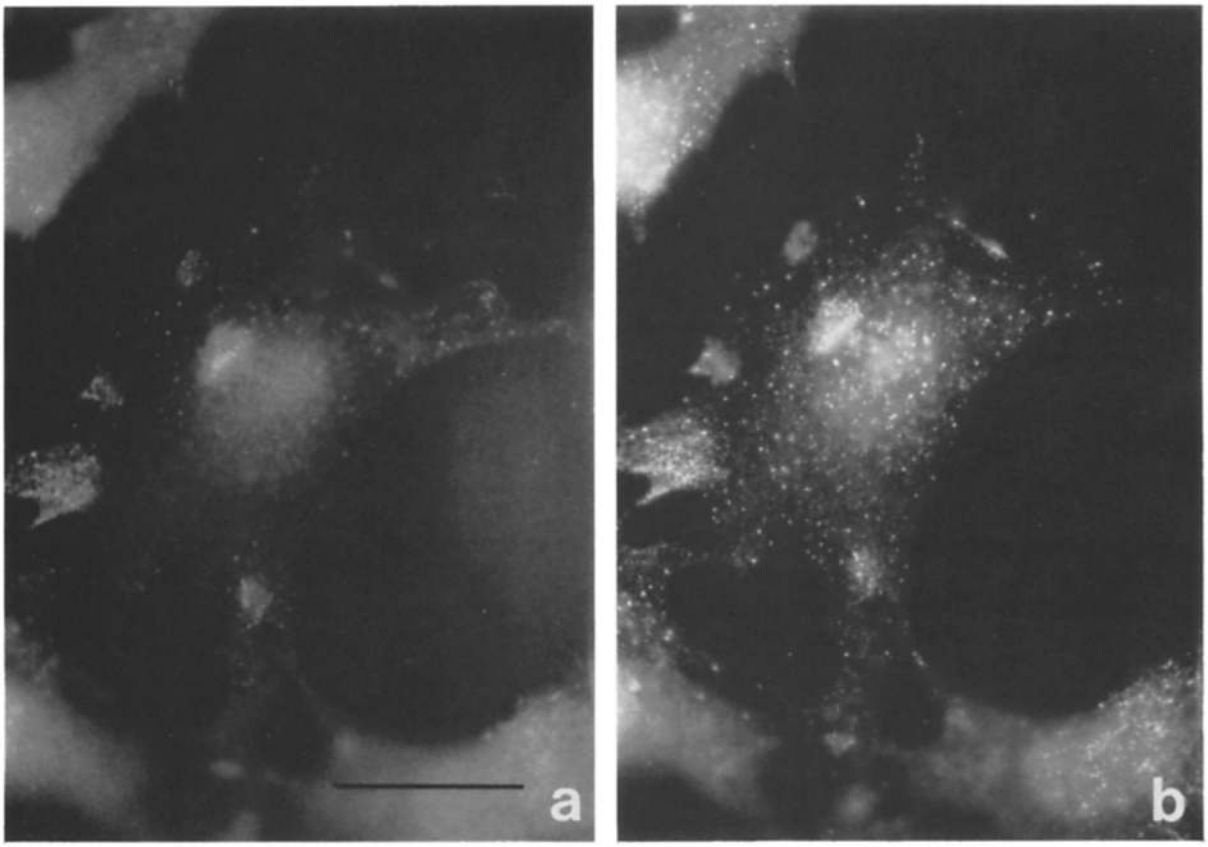

Figure 3. Immunofluorescent localization of encoded protein relative to HARC proteins. Double immunofluorescence studies of zinc-induced fibroblasts were performed using (*a*) mAb 3T3-5 which specifically reacted with 52–58-kD proteins of HARC and (*b*) pAb to peptide II (nucleotide sequence 804–864). The mAb was detected with FITC anti-mouse IgG and the pAb was detected with RITC anti-rabbit IgG. Both antibodies were strikingly localized in the ruffles and processes of zinc sulfate–induced fibroblasts. Preimmune sera showed no immunofluorescence (data not shown). Bar, 16 μm.

coded in the cDNA (nucleotide sequence 804–864) was abolished by light trypsin treatment (Fig. 2 *c*). Fluorescence-activated cell sorter analysis of these cells showed quantifiable staining providing further evidence of a cell surface localization (data not shown). In fixed cells, where lamellae were well-preserved, the encoded protein was seen to strikingly accumulate in the ruffles and processes of H-*ras*–transformed cells (Fig. 3). Staining also occurred intracellularly in the perinuclear region (data not shown). This distribution is typical of molecules that regulate cell locomotion (1, 14). It is further noted that the pAb to peptide I colocalized precisely with mAb to the 58-kD HARC protein (Fig. 3).

Mutant H-ras Gene Regulates Expression of RHAMM

Zinc sulfate induction of a metallothionein-regulated mutant H-*ras* gene transfected into 10T1/2 fibroblasts (212 cells; reference 26) increased the expression of 58-kD proteins (Fig. 4 *A*; +, −) detected by pAb to peptide II (nucleotide sequence 804–864, Fig. 1). p21 *ras* proteins were also increased upon induction (Fig. 4 *B*, +, −). An mRNA transcript of 5.2 kb was detected using a cDNA probe encoding the open reading frame of the 2.9-kb cDNA insert and was increased 24 h after zinc induction of p21 H-*ras* expression (Fig. 2 *c*). Northern blots were reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA for a loading control (Fig. 4 *D*).

Antibodies to Peptides Encoded in the RHAMM cDNA Block Locomotion

Direct evidence for a role of the encoded protein in H-*ras*–regulated cell locomotion was demonstrated in experiments designed to test whether antibodies to the encoded protein inhibited cell locomotion. As noted previously (34), induction of the mutant H-*ras* gene with zinc sulfate activated an HA-dependent motility mechanism in mutant H-*ras*–transfected fibroblasts (Fig. 5). Antibodies to peptide II (nucleotide sequence 804–864, Fig. 1) specifically inhibited *ras*-regulated locomotion (Fig. 5).

Bacterially Expressed RHAMM Binds to HA

An insert containing the open reading frame from the second initiation codon was expressed in bacteria as a glutathione-S-transferase fusion protein. The fusion protein was analyzed as described in Materials and Methods and separated from bacterial proteins by electrophoresis on 12.5% SDS-PAGE. RHAMM was identified with mAb 3T3-5, specific to the 52- and 58-kD HARC proteins, on immunoblots as a 75-kD protein (Fig. 6). About 26 kD is due to the presence of the glutathione-S-transferase peptide with the remaining 45–50 kD representative of the recombinant RHAMM peptide. The molecular mass of the recombinant peptide is therefore in agreement with that observed for the deglycosylated 58-kD protein isolated from fibroblasts (Turley, E. A., K. Hoare,

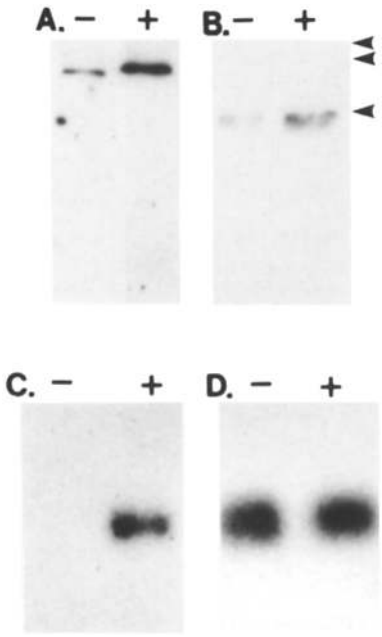

Figure 4. Expression of encoded protein is regulated by the mutant H-*ras* oncogene. Cell lysates (*A* and *B*) were prepared from buffer- (−) and zinc sulfate-treated (+) fibroblasts transfected with mutant H-*ras* under the control of a metallothionein promoter (26). Transblots from SDS-PAGE were probed with (*A*) pAb to peptide II (nucleotide sequence 804–864) or (*B*) RAS-10 antibody. Protein standards are marked by arrowheads and include, from the top of the gel, phosphorylase b (97.4 kD), BSA (68 kD), and trypsin inhibitor (21.5 kD). Induction of the mutant H-*ras* gene increased expression of the encoded protein. RNA (*C* and *D*) was isolated from buffer- (−) and zinc sulfate-treated (+) H-*ras*-transfected fibroblasts. RNA was hybridized with a ^{32}P-labeled cDNA fragment containing the open reading frame (*C*). Blots were reprobed with H-glyceraldehyde-3-phosphate dehydrogenase cDNA to control for RNA loading (*D*). The induction of the mutant *ras* gene with zinc sulfate increased the expression of a 5.2-kb mRNA transcript.

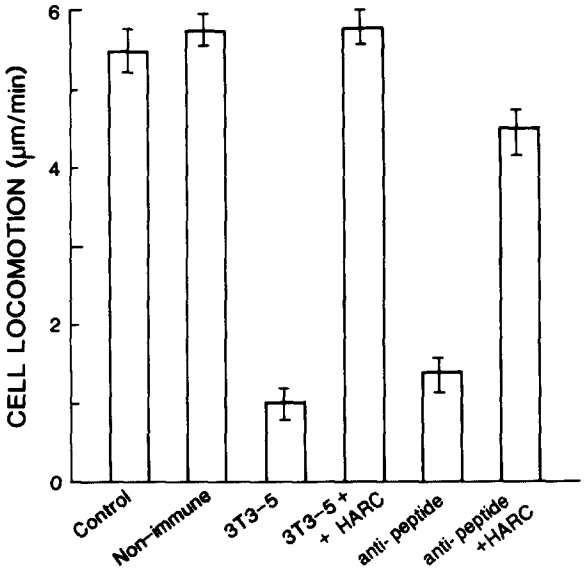

Figure 5. The encoded protein regulates *ras*-promoted cell locomotion. Mutant H-*ras*-transfected cells (26) induced with zinc sulfate were filmed by video timelapse and analyzed with a Dynacell program (Carl Zeiss, Inc.). The effect of nonimmune sera, mAb 3T3-5 to HARC and pAb to peptide II (nucleotide sequence 804–964) on HA-promoted locomotion was analyzed. Both mAb 3T3-5 to HARC and pAb to peptide II inhibited cell locomotion in response to HA. This effect was reversed by the addition of excess HARC proteins. Nonimmune sera had no effect on locomotion relative to controls. Values represent the mean + SEM. n = 50 cells.

and V. Cripps, manuscript submitted for publication) as well as that deduced from the nucleotide sequence. This protein specifically bound to biotinylated HA and was competed with excess labeled HA (Fig. 6). Bacterial lysates that contained plasmids without the insert did not bind HA (Fig. 6).

RHAMM Is Antigenically Related to the 58- and 52-kD HARC Proteins

The encoded protein was shown to be antigenically related to the 52–58 kD of HARC (32) which are the HA-binding proteins of this complex (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication). Thus, in transblot immunoassays and using purified HARC proteins as substrate (Fig. 7 *A*), PabI cross-reacted with the 58- and 52-kD proteins (Fig. 7 *C*). The blocking mAb 3T3-5 also cross-reacted with these proteins (Fig. 7 *B*).

Discussion

Oncogenic transformation by both *src* and activated *ras* genes have been reported to promote synthesis of HA (21, 32, 34) and the growth of many human tumors is accompanied by elevated levels of this glycosaminoglycan in the serum or in tissue surrounding the tumor (19, 25, 27). Tumor cells often show increased responsiveness to HA-stimulating factors (4, 13) and, recently, the increase in locomotion of a *ras*-transformed cell line has been shown to be mediated by HA (34). We have isolated and characterized a cDNA

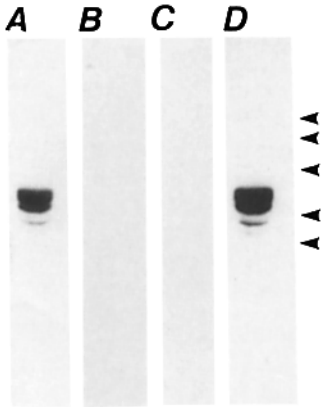

Figure 6. Bacterially expressed RHAMM binds to biotinylated HA. Lysates from induced bacteria plasmids with either an insert encoding RHAMM (lanes *A*, *B*, and *D*) or no insert (lane *C*) were electrophoresed on SDS–polyacrylamide gels and transblotted. Biotinylated HA was added to the blot in the absence (lanes *A* and *C*) or presence of 100-fold excess unlabeled HA (lane *B*). Bound HA was then detected with streptavidin-HRP and visualized with chemiluminescence. Transblots of bacterial lysates containing the insert were also incubated with monoclonal antibody 3T3-5 (lane *D*) to the 58- and 52-kD HARC proteins. These results show that HA bound to the expressed protein and binding was competed with unlabeled HA. mAb 3T3-5, which blocks locomotion, specifically recognized the expressed protein. The standards are marked with arrowheads and include α_2 macroglobulin (180 kD); β-galactosidase (116 kD); fructose-6-phosphate kinase (84 kD); pyruvate kinase (58 kD); and fumarase (48.5 kD).

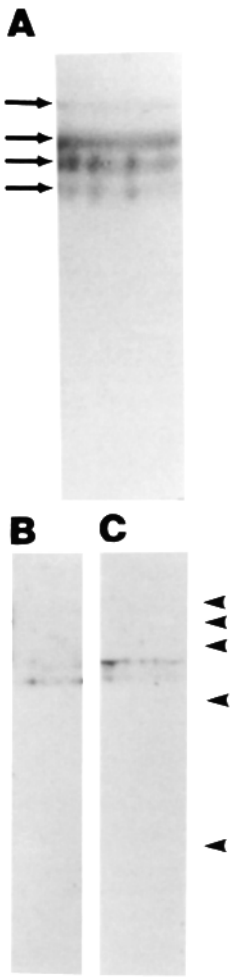

Figure 7. Immunoreactivity of anti-peptide antibody to the encoded protein. HARC proteins were purified from spent culture media, and then fractionated by SDS-PAGE and transblotted. HARC proteins were visualized with (lane *A*) India ink and had molecular masses of 72, 68, 58, and 52 kD (*arrows*). The 52–58-kD proteins reacted specifically with (lane *B*) mAb 3T3-5 (blocker of locomotion) to HARC, and (lane *C*) pAb to peptide I (nucleotide sequence 372–435). Molecular mass protein standards are marked by arrowheads and include from the top of the gel, myosin (200 kD); phosphorylase b (97.4 kD); BSA (68 kD); ovalbumin (43 kD); and trypsin inhibitor (21.5 kD).

clone from a λGT11 cDNA expression library prepared from 3T3 cells that encodes a 48- or 52-kD protein, depending upon the initiation codon used. This protein is unique, occurs on the cell surface, is regulated by the H-*ras* oncogene, and mediates locomotion of *ras*-transformed cells responding to HA. Furthermore, in transblot assays it specifically binds to HA. Its unique structure and HA-binding properties indicate that it is a new HA receptor. Further, its role in cell locomotion predicts that it will play an important role in developmental, disease, and repair processes. Based on these two functional properties, this protein is referred to as RHAMM, an acronym for receptor for HA-mediated motility.

The encoded protein appears to be identical or related to the 58- and 52-kD proteins of an HARC originally isolated by one of us (32). These proteins have been shown to be involved in locomotion (2, 33, 34) and, recently, to also bind to HA (Turley, E. A., K. Hoare, and V. Cripps, manuscript submitted for publication). Thus, pAb specific to the encoded protein cross-reacts with the 52, 58-kD HARC protein. Conversely, a battery of monoclonal antibodies that are specific for the 52- and 58-kD HARC proteins cross-react with bacterially expressed RHAMM. These observations, combined with (*a*) the ability of the HARC proteins to reverse the blocking effect of pAb specific to the encoded protein on cell locomotion (Fig. 5), (*b*) the precise colocalization of the two antigens, (*c*) their common regulation by the H-*ras* oncogene, and (*d*) their ability to bind to HA and to mediate *ras*-regulated locomotion provide strong evidence that the gene product isolated is identical to the HARC protein.

The HARC is released into the supernatant media (27) and contains several components that can be recovered by HA affinity purification, including 72-, 68-, 58-, and 52-kD proteins (32). Interestingly, the 52-kD HARC protein is not observed in cell lysates but only in the released form. The cell surface form of the complex, which is held together by as yet unidentified mechanisms, appears to be transmembrane (29). Ultrastructural studies reported elsewhere localize HARC (29) to the cell surface but the cDNA encoding RHAMM does not encode a long enough hydrophobic region to allow it to span the membrane. Either RHAMM is associated with an unidentified transmembrane component of, for instance, the HARC complex, or two forms of RHAMM exist: a soluble and membrane-associated form. The first prediction would resemble the organization observed for some animal lectins (1) and the structure that is predicted for an elastin–laminin receptor complex in which the laminin and elastin-binding component binds to a transmembrane receptor (18, 35).

Although the cDNA encoding the 56-kD protein does not contain a strong signal sequence, it is clearly located at the cell surface, as demonstrated by immunofluorescent staining of live cells with antibodies specific to RHAMM. Although somewhat unusual, a similar lack of signal sequence has been noted for other cell surface receptors including lymphocyte Fc receptor for IgE (10), transferrin receptor (15), liver asialoglycoprotein receptor (9), and the high-affinity laminin receptor (18, 35). It is of course possible that we have cloned a soluble form of RHAMM and that a membrane form also exists. We will further investigate these possibilities.

Previous to this report, several HA-binding proteins, including CD44 (3, 23, 24), hyaluronectin/versican (11), aggrecan (7), and link protein (8, 16) have been described. These proteins share a homologous region containing the HA binding domain (8). The predicted sequence of the cDNA reported here is unrelated to these molecularly characterized HA-binding proteins and the encoded protein therefore likely represents a new type of HA receptor. It is perhaps relevant that the 21-amino acid repeat motif identified from nucleotide sequence 372–684 (Fig. 1) contains a series of amphipathic alpha helices (5). Similar structures may be important in the binding of proteins of the clotting cascade to a related glycosaminoglycan, heparin (12).

In summary, we have characterized a novel hyaluronan receptor that is directly involved in tumor cell locomotion. The regulation of its expression by the *ras* oncogene predicts that it plays an important role in oncogenesis and possibly morphogenesis.

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